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ORIGINAL PAPER

Topographical imaging as a means of monitoring biodegradation of poly(hydroxyalkanoate) films

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Abstract Poly(hydroxyalkanoates) (PHAs) are a class of bacterially-derived polymers that are naturally biodegradable through the action of extracellular depolymerase enzymes secreted by a number of different bacteria and fungi. In this paper we describe the development of topographical imaging protocols (by both scanning electron microscopy; SEM, and confocal microscopy; CM) as a means of monitoring the biodegradation of solution cast films of poly(3-hydroxybutanoate-*co*-3-hydroxyhexanoate) (P3HB/3HHx) and medium-chain-length (*mcl*-) PHA. *Pseudomonas lemoignei* and *Comamonas* P37C were used as sources for PHA depolymerase enzymes as these bacteria are known to degrade at least one of the polymers in question. SEM revealed the bacterial colonization of the film surfaces while CM permitted the comparative assessment of the roughness of the film surfaces upon exposure to the two bacterial strains. By dividing the total surface area of the film (A′) by the total area of the scan (A) it was possible to monitor biodegradation by observing differences in the topography of the film surface. Prior to inoculation, P3HB/3HHx films had an A′/A ratio of 1.06. A 24-h incubation with *P. lemoignei* increased the A′/A ratio to 1.47 while a 48- and 120-h incubation with *Comamonas* resulted in A′/A ratios of 1.16 and 1.33, respectively. These increases in the A′/A ratios over time demonstrated an increase in the irregularity of the film surface, indicative of PHA polymer breakdown.

Keywords Poly(3-hydroxybutanoate-co-3-hydroxyhexanoate) · Degradation · Scanning electron microscopy · Confocal microscopy · Topography

Introduction

Poly(hydroxyalkanoates) (PHA) are synthesized by many species of bacteria as carbon and energy reserve materials and are accumulated intracellularly in the form of inclusion bodies. The most common type of PHA polymer consists of 3-hydroxybutanoic acid (P3HB), but many dissimilar bacterial cells synthesize more complex polymers depending on the genetic makeup of the organism and the accessible carbon source. Depending on the length and/or complexity of the side chains, PHA are generally classified as either short-chain-length (*scl*-: with carbon chain lengths between 3 and 5 carbon atoms) or medium-chain-length (*mcl*-: with carbon chain lengths greater than 6 carbon atoms). Generally, specific organisms accumulate one type of PHA or the other. However, a small number of bacteria have been identified that are able to accumulate copolyesters of *scl*- and *mcl*- monomers [1–3].

The natural role that PHA plays in bacterial survival makes it crucial that producing strains have the capability to enzymatically break down these polymers intracellularly in order to reap the benefits of production in the absence of exogenous carbon sources [4]. However, PHA polymers do find their way into the environment either through the death and lysis of accumulating organisms or increasingly through regular garbage disposal. As PHA polymers gain a

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larger market share and find increasing industrial applications as "green" substitutes for petrochemical-based polymers, these materials will begin to occupy a larger percentage of the solid waste stream, making an understanding of the biodegradation process imperative to ensure absolute mineralization and complete life-cycle assessments. The presence of PHA polymers in the environment, regardless of origin, establishes an additional feedstock for microbes, provided appropriate enzymes are present for conversion into monomeric components that can be passed across the cytoplasmic membrane and used for survival. Extracellular PHA biodegradation is broadly dispersed among bacteria and fungi (not necessarily those that produce PHA); aerobic and anaerobic PHA-degrading microbes have been isolated from diverse environments including soil, compost, aerobic and anaerobic sewage sludge, fresh and marine water and air [5–10]. The universal feature of each of these organisms is the secretion of specific PHA depolymerase enzymes. These carboxyesterases (EC 3.1.1.75; active against *scl*-PHA and EC 3.1.1.76; active against *mcl*-PHA) are incapable of intracellular degradation but are active solely in the extracellular breakdown of PHA polymers. This mutual exclusivity is the result of the different biophysical states of PHA polymers inside (amorphous) and outside (crystalline) the bacterial cell. Some bacteria are known to secrete enzymes with broader biodegradation specificity and although for some, this may be the result of multiple PHA depolymerases, others may produce one depolymerase with broad substrate specificity [11]. In fact, the most intensively studied PHA-degrading bacterium, *P. lemoignei*, is unique in that it synthesizes at least 7 extracellular depolymerase enzymes (PhaZ1–PhaZ7) that are active solely against *scl*-PHA polymers. Interestingly, PhaZ1 through PhaZ6 are active against the crystalline form of *scl*-PHA [12–15], while PhaZ7 has been documented to be active against *scl*-PHA in its amorphous state [16]. In contrast, relatively few *mcl*-PHA depolymerase enzymes have been described to date and the only one that has been purified and studied at the molecular level was isolated from *P. fluorescens* GK13 [17].

In order to assess the ability of an organism to biodegrade extracellular PHA polymers, simple clear zone techniques have been developed. These techniques involve the suspension of PHA in solid media and, upon inoculation; those microorganisms that synthesize extracellular PHA depolymerases hydrolyze the suspended PHA to water-soluble products resulting in transparent zones around bacterial growth. By comparing the diameters and opacity of the clear zones, comparative measurements of degradation efficiency between two or more organisms can be made. In addition, The American Society for Testing and Materials (ASTM) has developed and approved a number of protocols for measuring the biodegradability of polymers both aerobically (ASTM D5271-02, D5338-98, D5988-03, and D6691-01) and anaerobically (ASTM D5511-02 and D5526-94) [18]. In many of these cases, especially in aerobic degradation, mineralization using respirometry is used to monitor biodegradation where the generation of carbon dioxide is measured upon exposure of the PHA to a controlled environment. In this paper we describe a simple but effective method to monitor PHA polymer biodegradation through visual means with scanning electron microscopy (SEM) and confocal microscopy (CM) after exposure to known extracellular PHA depolymerase producers.

Materials and Methods

Materials

Poly(3-hydroxybutanoate) (P3HB) was purchased from Aldrich Chemical Company (Milwaukee, WI), while poly(3-hydroxybutanoate-*co*-3-hydroxyhexanoate (P3HB/3HHx; under the trade name NODAX^{TM}) was supplied by Dr. Phillip Green (Procter & Gamble, Cincinnati, OH) and was composed (as determined by peak integration from a previously reported GC/MS procedure, see Ref. [19]) of 90% 3HB and 10% 3HHx. Medium-chain-length PHA (*mcl*-PHA) was synthesized in our laboratory using *Pseudomonas resinovorans* NRRL B-2649 (obtained from the National Center for Agricultural Utilization Research; NCAUR, ARS, United States Department of Agriculture, Peoria, IL) grown on caprylic acid (PHA-CAP). The PHA-CAP polymer was synthesized in a 2-L batch culture (BioFlo III Batch/Continuous culture fermenter, New Brunswick Scientific, Edison, NJ) using Medium E* (see Ref. [20] for media composition) according to a previously published method [21]. The PHA-CAP polymer was composed primarily of 3HHx (12 mol%), 3-hydroxyoctanoic acid (3HO; 73 mol%) and 3-hydroxydecanoic acid (3HD; 10 mol%). *Pseudomonas lemoignei* ATCC 17989 was purchased from the American Type Culture Collection (Manassas, VA) and *Comamonas* P37C was supplied by Dr. Steven Goodwin of the University of Massachusetts-Amherst. All media components in the form of simple salts were purchased from Sigma Chemical Company (St. Louis, MO) and all solvents were HPLC grade and purchased from Burdick and Jackson (Muskegon, MI).

Molar Mass Determination

Molar mass averages were determined by gel permeation chromatography (GPC). Polymer concentrations were

2.0 mg/mL for the PHA-CAP and 0.5 mg/mL for the P3HB and P3HB/3HHx polymers. A Styragel HMW 6E (Waters Corp., Milford, MA) column was used with a calibration curve derived from polystyrene standards (Polymer Labs Inc., Amherst, MA) with narrow polydispersities. Chloroform was used as the mobile phase at a flow rate of 1 mL/min and the injection volume in all cases was 200 μL.

Latex and Overlay Plate Preparation

Latex was prepared from PHA-CAP by dissolving 1 g of PHA-CAP in 300 mL of acetone and dripping this solution into 125 mL of deionized water under rapid stirring. The white PHA-CAP latex was isolated by rotoevaporation to a volume of approximately 100 mL to remove the acetone and autoclaved to sterilize. The PHA-CAP latex and a suspension of P3HB in water (1% w/v) were used to prepare overlay plates for quick determination of depolymerase activity. Polymer concentrations of both PHA-CAP in the latex and the suspended P3HB (in mg/mL) were determined by weight difference for use in calculating the number of mmoles of polymer repeat units to be included in the PHA overlay plates described below. Two mL of PHA-CAP latex or P3HB suspension was added to separate tared aluminum pans. These pans were placed in a drying oven at 80 °C overnight and each pan was allowed to cool to room temperature, reweighed and the polymer concentrations (N; mg PHA/mL) calculated. Overlay plates were prepared by adding 10 mL of sterile 2% agar suspension (for the media composition used for *P. lemoignei*; SM media, see Ref. [22], and for *Comamonas* P37C; Com media, see Ref. [23]) into sterile petri dishes and allowing them to solidify. The N values (from above) were used in the following equation to determine the volume (V; in mL) of PHA-CAP (M_n = 75,000 g/mol; M_w/M_n = 1.66) latex or P3HB (M_n = 668,000 g/mol; M_w/M_n = 1.93) suspension needed to produce solutions of 1% agar containing approximately 10 mmoles of polyester repeat units:

$$V = \frac{10\,\text{mmoles}\,L^{-1} \times X\,\text{mg}\,\text{mmole}^{-1} \times Y}{1,000 \times N}$$

where X is the molecular weight of a single 3-hydroxybutanoate (88 amu) or 3-hydroxyoctanoate (144 amu) repeat unit and Y is the total volume (in this case 20 mL) of solution to be produced. Then, V mL of sterile PHA suspension, 15 mL of sterile 1% agar/minimal medium solution and (5–V) mL of sterile minimal medium were combined, mixed thoroughly, 7.5 mL added to the surface of each overlay plate and the agar allowed to solidify.

Verification of Depolymerase Activity

To induce and verify depolymerase activity polymer films of P3HB and P3HB/HHx (0.25 g) were solution cast from chloroform into the bottom of separate 125 mL Erlenmeyer flasks and dried under a nitrogen stream. The resulting films were 0.3 and 0.2 mm thick, respectively. Each flask was autoclaved to sterilize. Upon cooling, 50 mL of filter sterile SM media (pH 6.8) or Com media (pH 6.6) was aseptically added to each flask. These flasks, including flasks containing no polymer film as a control, were inoculated with 2 mL of bacterial suspension from a nutrient-rich, overnight bacterial culture and allowed to grow at either 30 °C (*P. lemoignei*) or 37 °C (*Comamonas*) with shaking at 250 rpm (PHA-CAP polymer film was not included for testing, at least under the pre-culture conditions, because: (1) the depolymerase enzymes produced by *P. lemoignei* are known to be inactive against *mcl*-PHA polymers [24] and (2) it was difficult to solution-cast a sterile chloroform-free PHA-CAP film in the bottom of an Erlenmeyer flask as the autoclave temperature is significantly higher than the melting temperature for PHA-CAP (T_m = 55 °C) and after 15 min at 121 °C partially degraded PHA-CAP polymers resulted.) After 24 h, sterile paper discs (dia. = 6 mm) were aseptically dipped into the bacterial suspensions and carefully placed on the surface of PHA-CAP and P3HB overlay plates. Each plate was incubated at the appropriate temperature (see above) to allow those cultures that contained depolymerase activity against PHA-CAP and/or P3HB to produce clear zones around the paper discs. The diameters of the clear zones were measured each day for up to 10 days or until the clear zone diameters reached 30 mm.

Film Formation for Topographical Degradation Studies

PHA-CAP and P3HB/3HHx polymer films were solution-cast from a 50 mg/mL suspension in chloroform onto individually tared round glass coverslips (dia. = 12 mm) until completely covered (approx. 5 drops) and were allowed to air-dry (P3HB was not used because it does not result in uniform films upon solvent casting due to varying evaporation rates). After 24 h, each coverslip was reweighed and the weight of the polymer films calculated by difference. Each coverslip containing either PHA-CAP or P3HB/3HHx films was placed into a sterile well of a 24 well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) and 2 mL of an overnight bacterial culture (grown in the presence of a P3HB/3HHx polymer film, described in the previous section) was added to each well and allowed to incubate statically at the appropriate temperature for up to 120 h. Films were harvested at 24 h intervals by rinsing each with deionized water and fixing

them in a 10:1 ratio of 0.1 M HEPES:glutaraldehyde solution.

Topographical Imaging

Scanning Electron Microscopy

For SEM P3HB/3HHx sample films were dehydrated in a graded series of ethanol solutions before critical point drying from liquid carbon dioxide. These dry film discs were mounted onto carbon adhesive tabs (Electron Microscopy Sciences, Hatboro, PA) on sample stubs and sputtered with a thin layer of gold. Digital images of P3HB/3HHx sample surfaces were collected with a model Quanta 200 scanning electron microscope (FEI Company Inc., Hillsboro, OR) operating in the high vacuum, secondary electron imaging mode. The PHA-CAP polymer films did not withstand the process of critical point drying and therefore were not examined by SEM.

Confocal Microscopy

Duplicate film samples of P3HB/3HHx and PHA-CAP were mounted in microwell dishes (MatTek Corporation, Ashland, MA) and immersed in 1 mL aliquots of 2.5% glutaraldehyde-0.1 M imidazole buffer (pH 7.2). The films were stained by adding a 5 μL aliquot of 0.1% Nile Red solution (Sigma Chemical Co., St. Louis, MO; for a good reference on Nile Red staining of lipidic materials see Ref. [25]) and the stain allowed to partition into the films. Imaging was performed with a model IRBE optical microscope (Leica Microsystems Inc., Bannockburn, IL) coupled to a model TCS-SP confocal system. Optical fluorescence was excited at 488 nm and emission between 580 nm and 620 nm was collected in a series of optical sections. The roughness of the film surfaces was computed with the LCS Materials software package (Leica Microsystems Inc., Exton, PA) from topographical images of fluorescence and surface reconstruction images were made from maximum intensities.

Results and Discussion

In this study new applications of different microscopic techniques were developed to provide a quick method of determining comparative PHA biodegradation rates among organisms. By measuring differences in the roughness of film surfaces, it was possible to assess and compare initial rates of biodegradation between *P. lemoignei* and *Comamonas* which are known to biodegrade certain types of PHA polymers under suitable conditions [23, 24, 26, 27]. Both of these bacterial strains were evaluated in our laboratory to determine the relative effect of different PHA polymers on depolymerase induction and biodegradation. Then, using this information, solution-cast P3HB/3HHx and PHA-CAP films were exposed to each bacterial strain under static conditions and the biodegradation process monitored by topographical inspection of the film surface. The P3HB/3HHx and PHA-CAP polymers were selected for this study because both polymers result in uniform solution-cast films for surface analysis and PHA-CAP is simple to synthesize in the laboratory by recognized fermentation techniques.

In order to evaluate the effects that polymer-type had on biodegradation, both bacterial strains were grown separately in liquid culture in the presence of P3HB (an *scl*-PHA polymer) or P3HB/3HHx (an *scl*-/*mcl*-PHA copolymer) films which were solution-cast on the bottom of separate culture flasks. After 24 h, the edges of the P3HB and P3HB/3HHx films were relatively non-uniform when compared to the control films that had been incubated in the absence of bacteria. Figure 1 shows P3HB/3HHx films after 24 h incubation in the presence of both *P. lemoignei* and *Comamonas* using an uninoculated culture as a control. It was apparent that in the absence of depolymerase-producing organisms, minimal film breakdown had occurred, as evidenced by the smooth, consistent edges of the P3HB/3HHx films. In contrast, the films that were incubated for 24 h with *P. lemoignei* and *Comamonas* were visibly more opaque and possessed edges that were noticeably more non-uniform. In fact, the P3HB/3HHx film incubated with *P. lemoignei* actually fragmented into four distinct pieces. These results showed that biodegradation had begun in both cultures.

In order to evaluate the effects of preculturing on the relative degradation rates of *scl*-PHA and *mcl*-PHA, sterile paper disks were dipped into each overnight bacterial suspension and placed on the surface of overlay plates containing either P3HB or PHA-CAP latex. After incubating the plates at the appropriate temperature (see Materials and Methods section) and measuring clear zone diameter over time, it was apparent that preculturing both organisms with P3HB/3HHx resulted in larger clear zones, evidence of enhanced depolymerization. Figure 2 shows the clear zone plates for both *P. lemoignei* and *Comamonas*. The presence of clear zones on the P3HB overlay plate inoculated with *P. lemoignei* (Fig. 2A) indicated the ability of *P. lemoignei* to biodegrade *scl*-PHA. The plate containing the *mcl*-PHA showed no clear zone formation (data not shown). This was entirely expected as it is well known that the depolymerase activity from *P. lemoignei* is specific for *scl*-PHA polymers. Interestingly, after four days of growth on P3HB overlay plates, the clear zones derived from P3HB/3HHx-precultured *P. lemoignei* were

Fig. 1 P3HB/3HHx films after 24 h preculture. The films were incubated either in the absence of PHA depolymerase-producing bacteria (control; **A**), in the presence of *P. lemoignei* (**B**), or in the presence of *Comamonas* (**C**). Note: notice the fragmentation of the film in the presence of *P. lemoignei* (**B**) and the jagged, irregular edge of the film incubated with *Comamonas* (**C**)

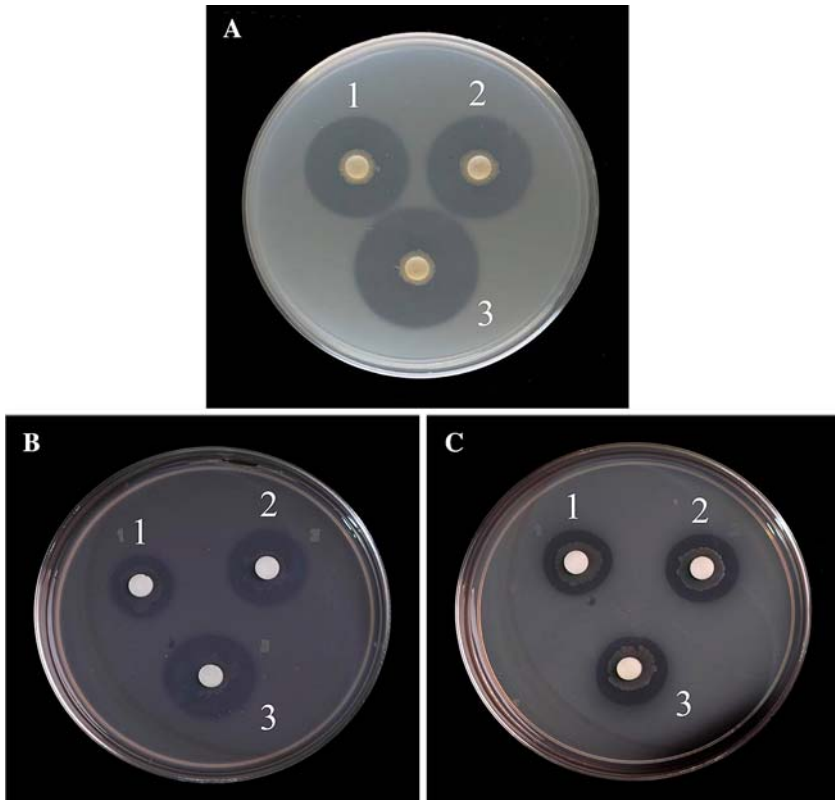

Fig. 2 Clear zone plates showing the degradation of P3HB and PHA-CAP by *P. lemoignei* (**A**) and *Comamonas* P37C (**B**, **C**) after having been precultured in the presence of no polymer film (1), a P3HB polymer film (2), or a P3HB/3HHx polymer film (3). Plates A and B contain powdered P3HB as the sole carbon source and plate C contains PHA-CAP latex as the sole carbon source. (For information on how the latex and the plates were prepared see the Materials and Methods section.)

14% and 16% larger than the clear zones derived from cells precultured on P3HB or in the absence of polymer, respectively. This showed that preculturing *P. lemoignei* with P3HB/3HHx stimulated extracellular depolymerization when compared to preculturing with P3HB or with no polymer film present. Whether this phenomenon is the result of crystallinity differences between the P3HB and P3HB/3HHx, compositional or molecular weight differences is unknown to date however, these questions may be the subject of future investigations.

The development of clear zones on both the P3HB overlay plates and the PHA-CAP overlay plates indicated that unlike *P. lemoignei*, *Comamonas* synthesized depolymerase enzymes that were active against both *scl*-PHA and *mcl*-PHA polymers (Fig. 2B, C). Clear zone formation with *Comamonas* was much slower than for *P. lemoignei*. In fact, after 4 days P3HB/3HHx-precultured *Comamonas* resulted in clear zone diameters that were 70% smaller than those from *P. lemoignei* against P3HB. After 8 days the clear zone diameters from P3HB/3HHx-precultured *Comamonas* had increased by 89% to 17 mm but were still 44% smaller than the clear zone diameters from *P. lemoignei* (diameter = 30.5 mm) after 4 days when precultured with P3HB/3HHx. In addition, *Comamonas* displayed the same trends as *P. lemoignei* with respect to clear zone diameters as P3HB/3HHx-precultured

Comamonas resulted in clear zone diameters that were 17% and 21% larger than the clear zones derived from *Comamonas* that were either precultured in the absence of polymer or precultured in the presence of P3HB, respectively. This seemed to indicate that under the experimental conditions used *Comamonas* was capable of but not as efficient at biodegrading P3HB as was *P. lemoignei*. *Comamonas* also showed effective depolymerization of PHA-CAP, at least in its latex form. It is unclear whether *Comamonas* synthesized multiple types of depolymerase enzymes or a single enzyme with broad substrate specificity, but what is clear is that after 8 days the clear zone diameters on the PHA-CAP overlay plates containing *Comamonas* were 10 mm (precultured in the absence of PHA polymer), 11 mm (precultured with P3HB) and 12 mm (precultured with P3HB/3HHx). These data point out that the effect of using P3HB/3HHx in a preculture with *Comamonas* diminished against *mcl*-PHA when compared to P3HB. Based on these results, the conditions used to preculture each organism prior to inoculating the P3HB/3HHx and PHA-CAP films for SEM and CM imaging were all performed in the presence of P3HB/3HHx.

Scanning electron microscopy was used to assess the surface topography of the P3HB/3HHx films both before inoculation and after incubation with each bacterial strain in order to evaluate the effects of each depolymerase on the film microstructure. Figure 3 shows P3HB/3HHx films prior to inoculation, after 48 h incubation in the presence of *Comamonas* and after 120 h incubation with *Comamonas*. It is evident that as time passed the films were broken down leaving a crescent-shaped film after 120 h. These crescent shapes were the result of slight thickness variations between the center and edge of the original films. Since these films were solvent cast, the evaporation rates varied slightly between the center and the edge of the films based on laboratory conditions. This resulted in films that showed a slight rippling effect as they dried (see Fig. 3A) making them slightly thicker along the edge and thinner towards their center. This slight variation in thickness caused the center of the films to breakdown sooner than the edge and resulted in crescent-shaped polymer films after exposure to the depolymerase-producing organisms. The same results occurred with *P. lemoignei*. However, the process in *P. lemoignei* was more rapid resulting in film fragmentation after 24 h (data not shown). The surfaces of the uninoculated P3HB/3HHx films were smooth. At magnifications of 250× the film was uniform in nature with no evidence of irregularities (Fig. 4A). The same films magnified to 2,500× revealed their microstructure which included very small cavities (average dia. ~1 μm) that uniformly covered the film surface (Fig. 4B). Because of their rapid biodegradation rates, the P3HB/3HHx films incubated with *P. lemoignei* were visualized after 24 h, at which time it was clear that pits (up to 50 μm in diameter) had developed on the surface of the film (Fig. 4C, D). Because of the high depolymerase production rate in *P. lemoignei*, it did not require a large number of bacterial cells to produce enough depolymerase enzyme to visibly disrupt the continuity of the film surface. In contrast, *Comamonas*, which degrades P3HB/HHx at a slower rate, began to show film surface irregularities after 48 h (Fig. 4E, G). These areas included locations where the organism had colonized the film surface. After 120 h, the process of biodegradation had progressed to the point where it looked as if the outer layer of the film surface had been removed leaving even larger irregular areas and increased bacterial colonization on the film surface (Fig. 4F, H). While SEM showed a detailed visual representation of the colonization and breakdown of the P3HB/3HHx film surface, it was not possible to quantify biodegradation simply from SEM pictures.

In order to use visual means to measure the comparative magnitude of biodegradation, we developed a CM technique to determine the roughness of the film surfaces after incubation in the presence of PHA degrading microorganisms. Confocal microscopy provided a method to visually monitor and compare the surface topography of the films over the course of the biodegradation process. By knowing the total area of the scan (A), and measuring the surface area (A′) of the film, relative roughness measurements of the film surface were calculated by dividing the surface area (A′) by the scanned area (A). An A′/A ratio of 1 suggests a surface that is absolutely flat, devoid of irregularities. Any increase in the A′/A ratio indicates an increase in the film surface roughness, which can be used as an indicator of the effectiveness of certain organisms in producing specific enzymes for the biodegradation of PHA

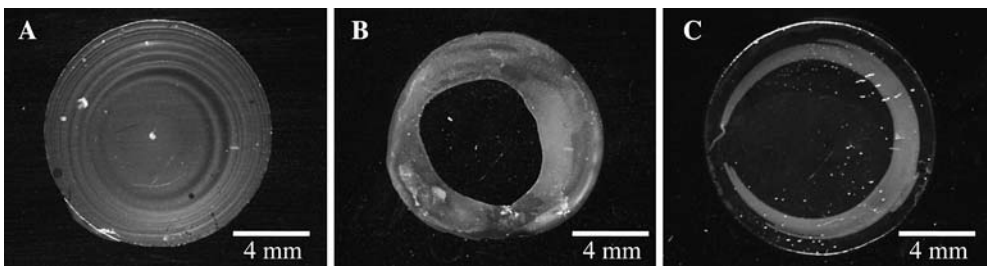

Fig. 3 Microscopic imaging of solution-cast P3HB/3HHx films prior to inoculation (**A**), and after 48 h incubation (**B**) and 120 h incubation (**C**) with *Comamonas* P37C

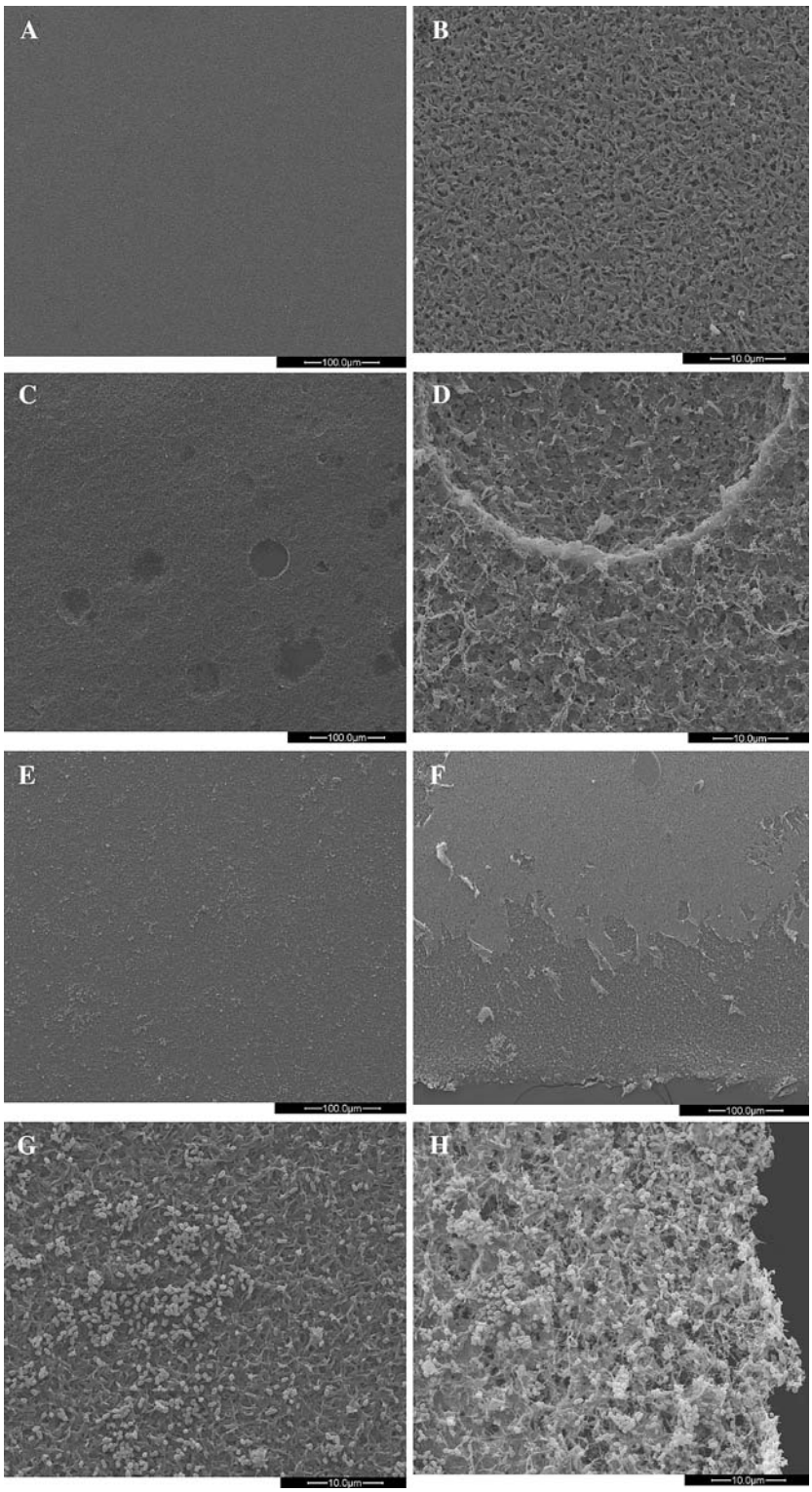

Fig. 4 Scanning electron micrographs of solution-cast P3HB/3HHx films prior to inoculation (**A**: magnification 250×, and **B**: magnification 2,500×), after 24 h incubation with *P. lemoignei* (**C**: magnification 250×, and **D**: magnification 2,500×), after 48 h incubation with *Comamonas* P37C (**E**: magnification 250×, and **G**: magnification 2,500×), and after 120 h incubation with *Comamonas* P37C (**F**: magnification 250×, and **H**: magnification 2,500×)

polymers. While this technique cannot be used to measure a reduction in film thickness or mass loss (additional experiments are necessary), it can aid in the determination of whether a particular organism may be an effective biodegrader of any polymer that can be molded into uniform films. By using this technique it was determined that the starting P3HB/3HHx polymer films had an A′/A ratio of 1.06 (Fig. 5A). At 24 h in the presence of *P. lemoignei*, the A′/A ratio increased to 1.47, and in fact, looking at the CM image (Fig. 5B) from a 90° perspective revealed the same

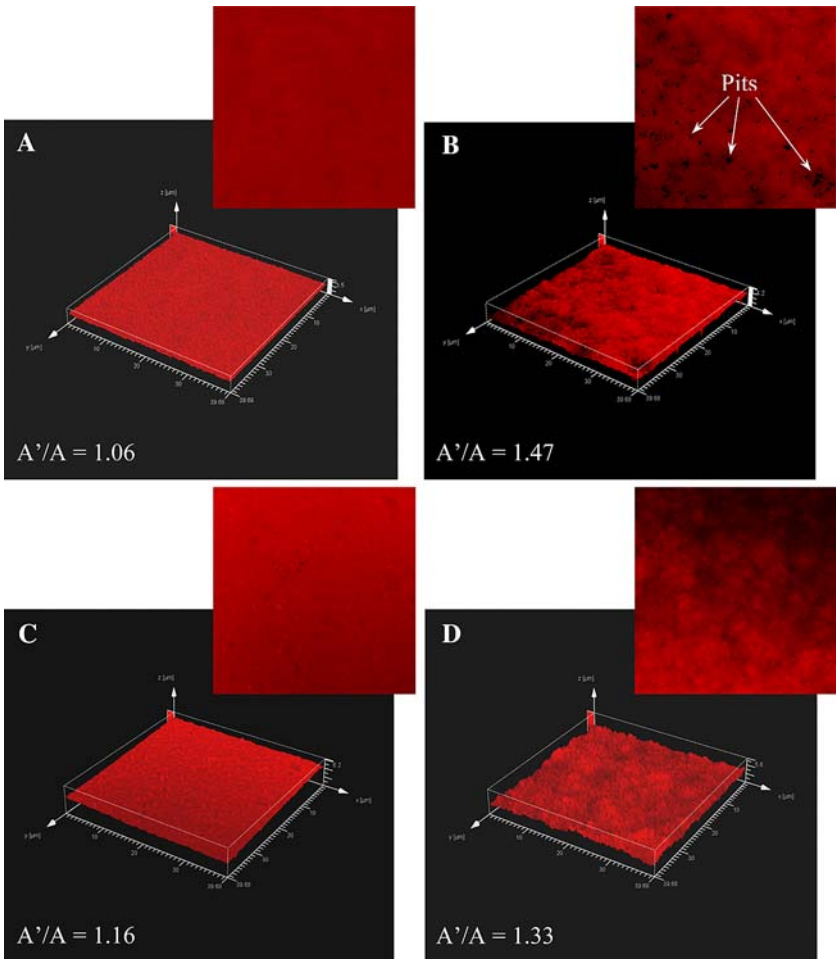

Fig. 5 Confocal micrographs of solution-cast P3HB/3HHx films prior to inoculation (**A**), after 24 h incubation with *P. lemoignei* (**B**), and after 48 h (**C**) and 120 h (**D**) incubation with *Comamonas* P37C. Note: The inset photographs show each film from a 90° perspective. (A′ = Total scanned area; A = Total surface area of the film)

pits in the film surface that had been seen by SEM. The consequence of the pits was to increase the surface area (A′) of the film, resulting in a larger A′/A ratio. *Comamonas* also resulted in an increase in the A′/A ratio, albeit at a reduced rate. In fact, after 48 h of static incubation with *Comamonas*, the A′/A ratio was 1.16 (Fig. 5C), increasing to a final value of 1.33 after 120 h (Fig. 5D). Increases in the A′/A ratio and in the "shadowing effects" on the film surfaces over time verified that this technique is beneficial for monitoring changes in the surface topography of polymer films. Based on the CM results, it is apparent that *P. lemoignei* is a better biodegrader of P3HB/3HHx than is *Comamonas*. However, it is important to remember that *P. lemoignei* only biodegrades *scl*-PHA while *Comamonas* is capable of biodegrading both *scl*-PHA and *mcl*-PHA. Based on this knowledge it seemed possible that the *P. lemoignei* system preferentially biodegraded the 3HB portion of the P3HB/3HHx film and left the 3HHx portion untouched while *Comamonas* at least partially biodegraded the 3HB and 3HHx together, which resulted in smaller A′/A values even up to 120 h, though biodegradation may have been rapid. In order to check this possibility, the polymer film weights from the *Comamonas* experiments were checked upon harvesting (Fig. 6). It was seen that after 1 day, 54% of the starting P3HB/3HHx film weight was lost. This value continued to climb to 89% after 5 days. Because topographical imaging is not capable of determining preferential degradation of one monomeric

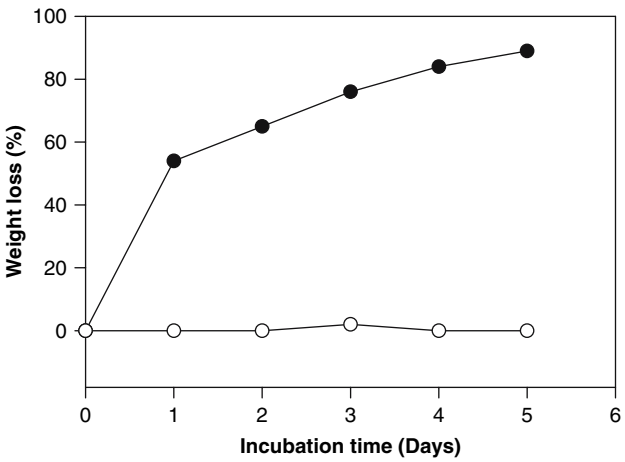

Fig. 6 Degradation results in terms of % weight loss of P3HB/3HHx (•) and PHA-CAP (○) films after incubation with *Comamonas* P37C

species over another, composition measurements of the residual polymeric material (by GC/MS; see Ref. [19]) were performed and revealed that the remaining intact polymer films were composed of 3HB and 3HHx in the same ratio (90:10) as the starting material. These results seemed to indicate that either both monomers were broken down at the same rate (unlikely as the *P. lemoignei* depolymerases are known to be specific for shorter chain PHAs) or that as the 3HB was degraded the 3HHx monomers were released into the environment and were no longer present as part of the polymer upon compositional analysis of the remaining film. Interestingly, the weight loss in the PHA-CAP film was virtually nonexistent. This was surprising based on the data from the overlay plates, which showed that *Comamonas* could indeed biodegrade *mcl*-PHA polymers. The physicochemical properties of a polyester including its stereoregularity, crystallinity, monomeric composition and polymer molecular weight, and the accessibility of the polymer surface all have a strong impact on its biodegradability to carbon dioxide and water. As latex, PHA-CAP was much more "available" to the organism because of the state of the polymer in aqueous solution. In its solid film state, PHA-CAP is more hydrophobic than in the latex or for that matter than P3HB. Therefore, even though it may come in contact with the fermentation broth containing the bacteria, the hydrophobicity of the polymer makes it more difficult for the depolymerase enzymes to act on the polymer surface which results in minimal to no biodegradation after 120 h. The PHA-CAP films had an A′/A ratio of 1.06 prior to inoculation with *Comamonas*. After 120 h the A′/A ratio was 1.05. These values by themselves could indicate that either the organism was able to biodegrade all of the monomers at identical rates or that nothing happened. The lack of weight loss in the PHA-CAP films over time indicated that no biodegradation had taken place up to 120 h (although longer durations may have helped the process) and that biodegradation rates were indeed higher with *P. lemoignei* than for *Comamonas*, at least against P3HB/3HHx films.

In conclusion, it has been demonstrated that the ability of any particular organism to degrade PHA polymers is dependent upon the environmental conditions including the specific depolymerase enzyme produced by the organism and the type of PHA present. In this paper topographical imaging techniques were developed (including SEM and CM) to assess the biodegradation of certain PHA films. It was determined that by monitoring the surface roughness of the polymer films, one could evaluate whether the films were effectively biodegrading based on the ratio of the surface area of the film (A′) to total scan area (A). Increases in this ratio signify the generation of a less uniform film surface, which may be indicative of biodegradation. This method does have limitations; it cannot determine preferential biodegradation of one monomer compared to another or mass loss over time. But, it can provide a new tool for the rapid determination of whether a particular organism has the capability to synthesize an enzyme targeted toward any polymer, provided that the polymers can be cast into films, and will retain a stain. This new procedure will aid in the study of biodegradation by providing a quick method for determination of the efficacy of certain depolymerase enzymes to the feasibility of polymer biodegradation.

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